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TECHNICAL NOTE

Eighteen SSR-primers for tetraploid *Adansonia digitata* and its relatives

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Abstract Co-dominant markers suitable for molecular ecological studies in the genus *Adansonia* are highly desirable in order to be able to address a number of interesting research questions related to the special life history traits, gene flow, and distribution dynamics of the *Adansonia* species. This note presents a set of 18 SSR-primers developed for *Adansonia digitata*, and tested for cross-amplification on all members of the *Adansonia* genus. All reported primers were found to amply loci with more than two alleles per locus in *Adansonia digitata*, reflecting its tretraploid nature. Segregation of alleles in open pollinated progenies from trees with four alleles per locus indicated polysomic inheritance for 14 of the 18 loci.

Keywords *Adansonia digitata* · Baobab · Microsatellites · SSR · Segregation · Crossamplification · Polyploid

Introduction

The African baobab, *Adansonia digitata* L., is a spectacular and intriguing tree species due to its autecology, African-wide distribution and large socio-economic importance. From a biological point of view this pachycaul has been denoted the worlds largest succulent with the ability to grow more than 1000 years old (Patrut et al. 2007). Its trunk is capable of absorbing water to an extent so the circumference of the trunk expands several percent. Being leafless except 2 to 3 months a year, it is assumed that considerable photosynthesis is taking place in the bark. Culturally, the tree is subject to many myths and superstitions (Wickens and Lowe 2008).

Adansonia includes seven additional species that share many morphological and biological characteristics with their African relative. They appear, however, to have only half the number of chromosomes (Baum and Oginuma 1994). Six of them are native to Madagascar (*A. za* Baill., *A. madagascariensis* Baill., *A. rubrostipa* Jum.& H Perrier, *A. suarezensis* H. Perrier, *A. perrieri* Capuron, *A. grandidieri* Baill.) and one to Australia (*A. gregorii* Mueller).

The large flowers of *A. digitata* are open for a single night per flower and are predominantly pollinated by fruit bats (Baum 1995a). Little is known about the foraging patterns of Old World fruit bats, and thus the pollination of baobabs (Andriafidison et al. 2005). Gene flow studies will thus serve the dual purpose of providing insight into the dispersal processes that shape the genetic structure of the baobabs as well as providing indirect information on fruit bat foraging behavior. Also, estimates of seed dispersal and differentiation between populations will be very useful for monitoring impacts from anthropogenic influence and for predicting consequences of climatic fluctuations. This is particularly important since several Malagasy species have long been known to be threatened, and the African baobab seems to be declining due to heavy browsing and human activities, especially in East and Central Africa (Baum 1995b; Jama et al. 2008; Wilson 2008). The phylogeography of *A. digitata* has long intrigued researchers

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with its present disjunct distribution and anthropological indices of late arrival to its western distribution range (Wickens 1982). Use of a combination of marker types might be able to provide an explanation (e.g., Dick and Heuertz 2008).

Polyploid species cause some difficulties for the molecular geneticist. Direct sequencing is more difficult compared to diploids, and scoring of co-dominant markers is challenging. This is especially true where one needs to know dosages and predict segregation as is the case of paternity studies. Segregation provides a challenge, because of the difficulties in unraveling poly, disomic, or mixed inheritance. For polysomically inherited loci, the issue of double reduction further complicates things. When it comes to even simple computations, there is also a lack of software dealing with these omnipresent polyploids, whereas more advanced analyses and algorithms are almost non-existent.

Materials and methods

Individuals for development and testing of the present SSR library were located in and around the village of Nobéré, Burkina Faso (11°33.900′N 1°12.300′W). Bark samples for DNA extraction were taken out using a bark blazer, and samples were field dried in silica gel. DNA was extracted using Qiagen DNEasy Plant Mini Kit. A microsatellite-enriched library was built following the protocol of Billotte et al. (1999). Total DNA was digested using *Alu* I (New England Biolabs). Adaptors Rsa21 and Rsa25 (Edwards et al. 1996) were ligated to the fragments using T4 ligase (Roche). Enrichments were done for the motifs $(TG)_{10}$, $(TC)_{10}$, $(TTA)_8$, $(TGA)_8$, and $(TTC)_8$ following the protocol of Glenn and Schable (2005) by use of Streptavidin MagneSphere Paramagnetic Particles (Promega). Enriched DNA was PCR amplified and cloned using the pGEM-T Easy Vector System Kit (Promega). Following screening based on the β-galactosidase gene, 288 plasmids were isolated. A PCR-based test for insert presence and size resulted in 192 plasmids of length between 500 and 1,000 bp. These were sequenced in both orientations at Macrogen, Korea using a 3730xl sequencer (ABI).

Primer sequences were designed for 32 loci. From these, 18 were selected based on ability to amplify, level of polymorphism, and interpretability. Testing was carried out on a sample of 214 individuals of *A. digitata*, and 30 individuals of the seven remaining A*dansonia* species (Table 2). Tests were carried out in 10 µl reactions using Qiagen Multiplex PCR kit following the manufacturer's instructions. For testing, a T_A of 58°C was used for all loci.

Results and discussion

All of the 18 reported loci were found to be highly polymorphic and very simple to score (Table 1). SSRs based on tri-nucleotide repeats were found most easy to score and could be subjected to auto-detection of alleles, whereas di-nucleotide repeats with long bp-ranges need manual scoring due to low signal of the longer alleles.

Looking at 20–25 offspring genotypes from each of 30 mother trees with open pollination (unpublished data), it was possible to infer on the inheritance of the loci. In situations where maternal genotypes included four different alleles, the inheritance could be inferred by looking at progenies with four different alleles of which only two were shared with the maternal genotype. For such mother-offspring arrays, polysomic inheritance could be inferred if any given maternal allele had paired with each of the remaining three in the maternal gametes. If the offspring had less than four different alleles, double reduction could not be ruled out as an explanation for the seemingly random pairing of alleles. However, double reduction would still indicate polysomic inheritance at the given locus. Using the first approach, loci Ad04, Ad06, Ad07, Ad11, Ad14, and Ad14 were inferred to be polysomically inherited. Expanding to look also at offspring that had less than four alleles, polysomic inheritance was inferred for all loci except Ad09, Ad10, Ad12, and Ad13. More thorough testing will be possible once it has been established to what extent the allele peak area can be used to estimate the allelic configuration (e.g., Catalán et al. 2006). It is suggested to test this by analysis of offspring from controlled test crosses between individuals with selected genotypes (e.g., Stift et al. 2008).

High transferability of primers within the genus was observed as is evident from Table 2. Having half as many chromosomes compared to *A. digitata*, the remainder of *Adansonia* species were expected to have no more than two alleles per locus. However, some primer pairs were found to give three or four bands when amplifying certain individuals. This was consistent even at higher annealing temperatures. The reason for this is unknown but it could be speculated that it is caused by amplification of loci in multicopy-DNA or genomes from an ancient polyploidization event. As would be expected, some primers did not amplify in all the species. In addition, some primers did not amplify in all individuals in a species. The latter is expected to be due to a poor match between primer and priming site, and might be possible to fix by redesigning the primers. Further work on testing transferability and verification of priming sites is encouraged.

Table 1 The reported SSR loci listed with GenBank accession numbers and forward and reverse primer sequences

Locus name	GenBank Accession no.	Primer sequences 5′–3′		Motif	Size range (bp)	N_A	Peaks per individual (fraction)			
		F	R				*p*(1)	*p*(2)	*p*(3)	*p*(4)
Ad01	GQ303181	CATTGCCAGGAATGCTTTTGC	GGATTGCCAGGTCTACTAC	$(AG)_{19}$	96–125	8	0.24	0.61	0.15	0.00
Ad02	GQ303182	TGCTGACTAGCAGTTTCCTATG	TCAGATGCCAAACATTCACACC	$(TC)_{15}$	267–295	8	0.07	0.42	0.40	0.12
Ad03	GQ303183	GGATCAAATTATGGTTAAGGC	CCAATTTTGAGCCAATTCTCA	$(GA)_{21}$	143–175	9	0.05	0.47	0.40	0.08
Ad04	GQ303184	GTTGCTTGTGTGCTTACCC	CATCCCTCTCCCCATTCC	$(CT)_{20}$	173–236	27	0.19	0.46	0.26	0.10
Ad05	GQ303185	CTCAACAAGGTTCGGATGTCGTATG	GTCTGCCGGGTGTTTTGCATG	$(CA)_{12}(CT)_{12}$	295–319	10	0.12	0.50	0.32	0.06
Ad06	GQ303186	TGCATCAGCTTTCACTCCAGAC	GCCACCCATAAAACCCAATCC	$(TC)_{19}$	129–154	8	0.01	0.21	0.56	0.22
Ad07	GQ303187	TAGAAAATTAGCAGATAAGTGC	GATTTCGGTGATATGTTGTAG	$(AG)_{18}$	159–193	17	0.00	0.18	0.55	0.27
Ad08	GQ303188	TCTAAAGCCTGTAAGGAAAAATGGG	TTCTCCGTTCACTCTGTACTTCC	$(GAA)_{14}$	267–296	11	0.03	0.29	0.52	0.15
Ad09	GQ303189	TACCACTTCTCCAGATGCTAC	ACTGGCTAGAGATGCGTTG	$(AAG)_{11}$	190–209	5	0.04	0.46	0.45	0.06
Ad10	GQ303190	GCAGCTTGCCTCGTCATATA	CCAATGGCAATGTGTCTGACG	$(CT)_6CC(CT)_{19}$	216–262	7	0.42	0.47	0.09	0.01
Ad11	GQ303191	ATCAGCCATTCTGCATACCTGC	TAGGCACAAAACTGAGATGCACAG	$(CA)_{13}(AT)_6$	118–181	26	0.01	0.09	0.46	0.44
Ad12	GQ303192	GCTTGTCAAGCAATTCCCC	ACTTTGTCCCACCTGTTTCTC	$(AG)_{16}$	162–175	5	0.19	0.47	0.31	0.03
Ad13	GQ303193	CCCCACTTCAGATCAAGTAAGTC	GCTGTATTTCTGAGCCTGAGAAG	$(AC)_{14}$	305–330	11	0.01	0.20	0.57	0.21
Ad14	GQ303194	CTTGATTGGAATACGGGAAATGGAG	CCAAACCAATTGGACTTTGACCTTC	$(AC)_{13}$	170–191	10	0.00	0.12	0.51	0.36
Ad15	GQ303195	TGAAGAGACAAAGCAAGAAG	CATGACATCTCCTTGAACC	$(GAA)_{14}$	130–161	9	0.13	0.41	0.43	0.03
Ad16	GQ303196	TGCATCAGCTTTCACTCCAGAC	GCCACCCATAAAACCCAATCC	$(TA)_5(TG)_{19}$	219–254	14	0.01	0.21	0.54	0.24
Ad17	GQ303197	GCGCCTTAGAAAGGACTTGTTAGAG	GCCAACAGCCTTAGTAGTCCAAG	$(AC)_{14}$	174–215	22	0.01	0.21	0.51	0.27
Ad18	GQ303198	ACCGCTTCCGTTCTCATTCC	ACCACCACTACACCGTCATTG	$(TG)_{17}$	257–291	18	0.14	0.40	0.37	0.09

Size range gives the interval in base pairs for which alleles were found in the studied population. N_A gives the number of alleles found for each locus, and *p*(1)–*p*(4) gives the fraction of genotypes with 1, 2, 3 and 4 different alleles, respectively. Diversity was estimated based on 214 genotyped individuals

Table 2 Results from the transferability test

Species	Section Brevitubae		Section Longitubae				
	A. gran.	*A. suar.*	*A. greg.*	*A. za*	*A. perr.*	*A. rubr.*	*A. mada.*
N	3	2	11	5	3	2	4
Ad01	+	+	+	+[a]	+	+	+[a]
Ad02	+	+	+	+	+	+	+
Ad03	+[b]	−	+	+[a,b]	+[b]	+	+[b]
Ad04	+	+[b]	+[a]	+	+[b]	-	+[b]
Ad05	+	+	+[b]	+	+[a]	+	+
Ad06	+	+	+	+[a]	+	+	+[a]
Ad07	+	+	−	+	+[b]	+	+
Ad08	+	+	+	+	+	+	+
Ad09	+	+	+	+	+	+	+
Ad10	+	+	+	+[b]	−	+	+[b]
Ad11	+[a]	+	+[a]	+	+[a]	+	+[a]
Ad12	−	−	+	+[b]	+[b]	−	−
Ad13	+	+	+	+	+[b]	+	+
Ad14	+	+	+	+	+	+	+
Ad15	+	+	+	+	+[a]	+	+
Ad16	+	+	+	+	+[a]	−	+
Ad17	+	+	+[b]	+[b]	+[b]	+	+[b]
Ad18	+	+	+	+	+[b]	+	+

Amplification (+) and no amplification (−) is given for each combination of locus and species. *N* gives the number of individuals tested, superscript letter (a) denotes that one or more individuals had more than two bands for the given locus and superscript letter (b) denotes that one or more individuals did not amplify

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